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Production and Charaterization of Amylase Enzyme by Aspergillus Niger Isolated from Rhizospheric Soil Under Solid State Fermentation and Submerged Fermentation

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ABSTRACT:

This paper focuses on the isolation and production of amylase by Aspergillus niger from soil using twodifferent fermentation methods: Submerged Fermentation (SmF) and Solid-State fermentation (SSF). The aimof this study is to compare the efficiency and yield of amylase production between these two methods.Additionally, produced crude amylase will be characterized based on its performance under different pHlevels, temperatures, and thermal stability.The SSF method involves the growth of *Aspergillus niger* on a solid substrate, while the SmF method utilizes liquid medium for the growth of the microorganism. The production of amylase has been monitoredandcompared in terms of enzyme activity and yield.

To further understand the properties of the newly produced amylase, various characterization experiments hasbeen conducted. Different pH levels will be tested to determine the optimal pH for enzyme activity. Similarly,a range of temperatures has been evaluated to identify the temperature range in which the amylase exhibitsmaximum efficiency. Thermal stability experiments has been performed to assess the enzyme's ability towithstand high temperatures without losing its activity.This dissertation aims to contribute to the understanding of amylase production by *Aspergillus niger* andprovide valuable insights into the characterization of the newly produced enzyme. This study finding have implications for industrial applications, such as the development of more efficient and stable amylasebased processes.

Introduction:

The production of enzymes through microbial fermentation has gained significant attention in various industries, including the food, pharmaceutical, and biofuel sectors. Among these enzymes, amylase plays a crucial role in the hydrolysis of starch, making it a valuable biocatalyst for numerous applications. *Aspergillus niger*, a filamentous fungus, has been widely studied for its ability to produce amylase through both solid-state fermentation (SSF) and submerged fermentation (SmF) methods.SSF involves the growth of microorganisms on a solid substrate, such as agricultural residues or industrial by-products, providing a cost-effective and environmentally friendly approach for enzyme production. On the other hand, SmF utilizes a liquid medium, allowing for better control of process parameters and scalability. Understanding the differences in amylase production between these two fermentation methods can provide valuable insights into optimizing enzyme production processes.Characterizing the newly produced amylase is essential to determine its potential applications and optimize its performance. Factors such as pH, temperature, and thermal stability greatly influence the enzyme's activity and stability. Evaluating the enzyme's performance under different pH conditions can help identify the optimal pH range for maximum activity. Similarly, studying the effect of temperature on amylase activity can provide insights into its temperature range for efficient catalysis. Furthermore, assessing the enzyme's thermal stability is crucial for applications that require high-temperature processes.This dissertation aims to investigate the production of amylase by *Aspergillus niger* through SSF and SmF methods, as well as the characterization of the newly produced amylase based on different pH, temperature, and thermal stability. By understanding the factors influencing amylase production and its performance, this research can contribute to the development of efficient and sustainable enzyme production processes, as well as the identification of potential applications in various industries.

Keywords: Amylase, *Aspergillus niger* , Fermentation ,Submerged Fermentation (SmF), Solid-State Fermentation (SSF), Enzyme production , Comparative analysis

Materials and Methods:

I. Isolation of Microorganism from soil sample:

i) Preparation of 5 Potato Dextrose Agar(PDA) plates and 5 Nutrient Agar (NA) plates and autoclave the media for 30 min.

ii) Rhizospheric soil has been collected from garden, a set of serial dilution was done(10^{-1}-10^{-6}) after taking 1gm. of soil and 10ml sterile distilled water (stock). 0.1 ml of diluted soil suspension from 10^{-3},10^{-4}, 10^{-5} dilution were taken and spread on 3 sterile PDA and 3 sterile NA petri plates. Growth was found in NA plates after 48 hours of incubation at 37^0C in 10^{-3} dilution PDA plates and NA plates.

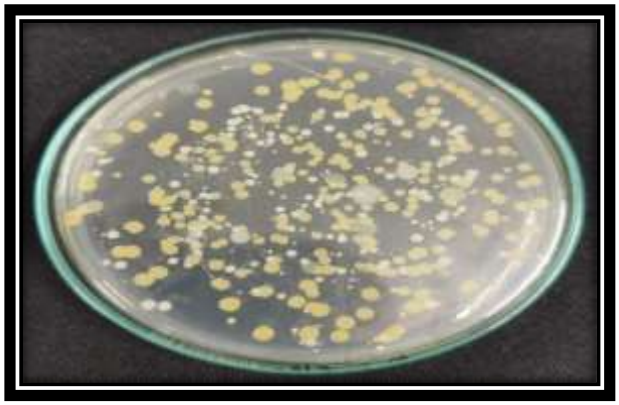


Figure 1:NA plates of 10^{-3}dilution

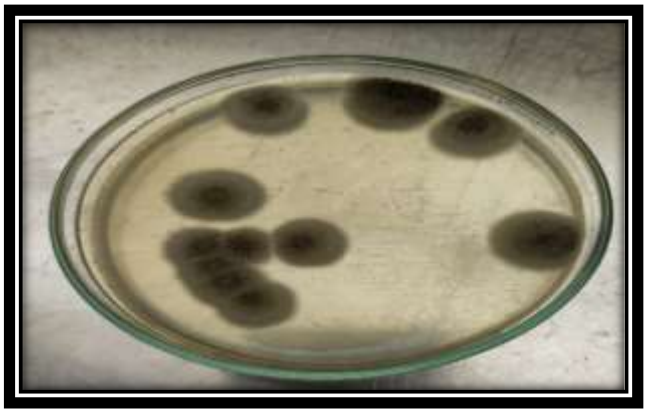


Figure 2: PDA plates 10^{-3}dilution

iii) Streaking were done by picking white colonies in 2 Starch Agarplates, yellow colony in 1 Starch Agar plate from NA platesand incubated at 37^0Cfor 48 hrs.Fungal colonies were streaked in 2 SA plates from PDA plates and incubated at 30^0C for 72 hrs.

iv)Then fungal colonies were again inoculated to PDA plates and 6 PDA slants to get pure culture.

Figure 3: PDA plate

Figure 4: PDA slants

II. Screeningand identification of Amylase producing Aspergillus Niger : Identification of *Aspergillus niger*: After the fungal colonies have grown, visually inspect the plates for colonies that appear characteristic of *Aspergillus niger*. *Aspergillus niger* colonies typically exhibit a velvety or powdery texture with a dark green to black color. However, visual identification alone is not sufficient for accurate identification.

Microscopic Examination: The fungal colonies were microscopically examined using Lactophenol cotton blue and observed under compound microscope at 40X . *Aspergillus niger* typically produces conidiophores with conidial heads that are columnar and radiate outwards in a characteristic brush-like or broom-like arrangement.

Amylase screening: After the fungal colonies appear, we perform a preliminary screening for amylase production using a starch agar plate. Starch agar contains starch as the substrate, and the amylase-producing strains will exhibit a clear zone around their colonies due to starch hydrolysis.

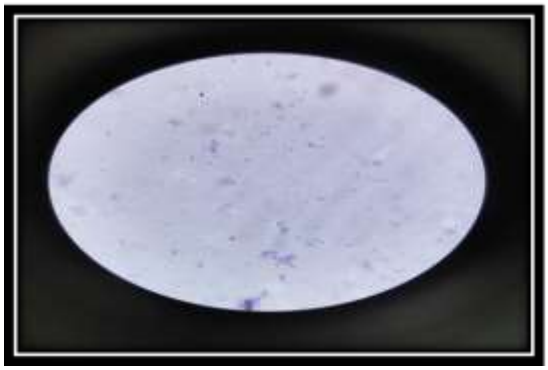


Figure 5: Microscopic view

Figure 6:Identification of amylase: clear zone

The most commonly used identification method is the iodine-starch method. The enzyme hydrolyzes starch, resulting in the formation of soluble fragments, which do not react with iodine, leading to a decrease in the blue color and producing a clear halo zone.

a) Solid state fermentation:- Media preparation- 1)wheat bran - 5g for 100ml media , 2) Basal salt solution 100ml (50ml+50 ml) for 2 set - (NH2)2SO4 - 0.2 g ,KH2PO4 - 0.1g, MgSO4.7H2O – 0.05g , ZnSO4 – 0.01g 3)Two 250ml conical were taken for 2 set where we added 50 ml of basal salt solution in both conical, then 5g of wheat bran in each of two conical were added. After that sterilize the solution by autoclaving for 15 min. 4)1% of inoculum from slant were added after cooling the media. And incubate at 300C for 4 days.

b) Submerged fermentation: -

Media preparation- for 200 ml media for 2set (100ml of each set) – KH2PO4 – 2.8g, (NH4)2SO4 – 2g, KCl – 1g, MgSO4.7H2O -0.2g, FeSO4.7H2O – 0.02g (dried). In two 250ml conical for 2 set 100 ml basal salt solution were added. Then 1.5g of starch were added into both conical. Maintain the pH 6. After preparing the media inoculum were added from slant culture into both conical and incubated them at 120rpm for 48 hours into incubator(300^0C).

Extraction of crude amylase:-

For SSF 100ml of Tris was buffer added in both conical ,then put into shaker for 1 hour at 130 rpm , filtered it and collected the crude enzyme.

Figure 7 : Adding inoculam to media

Figure 8 : After 4 days incubation

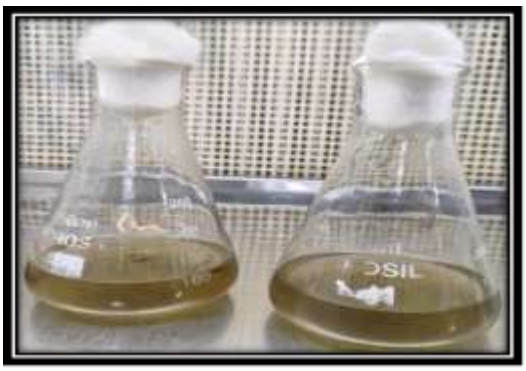


Figure 9 : Adding culture to media

Figure 10 : 48 hours Incubation

Figure 11: Filtration of crude enzyme

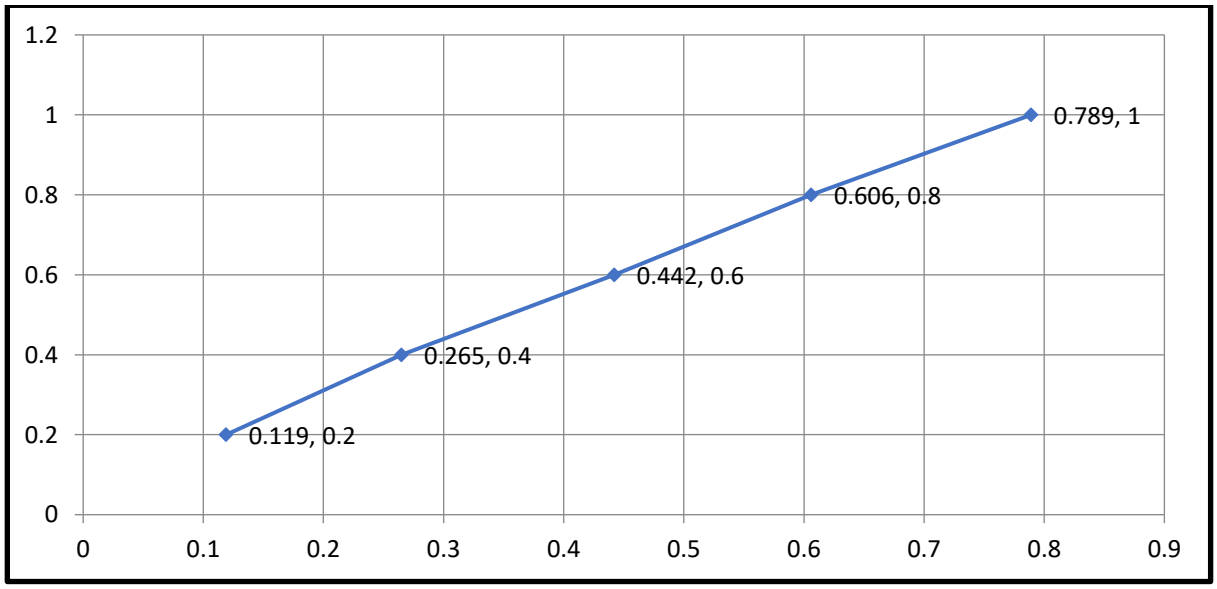
Figure 12 : Filtered crude enzyme (amylase)

Estimation of Amylase activity:-

To estimate amylase activity, the standard curve of Maltose of known concentration were done using stock maltose =1mg/1ml (taking 6 test tube we added stock maltose into 5 test tube , and one test tube will be blank. Then we added 1ml of water and 1ml of Dinitrosalicylic acid(DNS).after adding DNS waterbath the test tubes for 5-10 min.after cooling the test tube we took the OD at 540nm.).

Standard curve of maltose :-

	Maltose conc.(mg/ml)	**Maltose volume(mg/ml)**	**Water added (ml)**	**DNS added (ml)**	**OD (540nm)**
Blank	----	----	1	1	0
1	0.2	0.2	0.8	1	0.119
2	0.4	0.4	0.6	1	0.265
3	0.6	0.6	0.4	1	0.442
4 5	0.8	0.8	0.2	1	0.606
	1.0	1.0	-----	1	0.789



Assay of amylase activity:-

For the activity of crude enzyme from SSF and SmF: 12 test tubes were taken. . For two set of crude enzyme SSF and SmF. So, one test tube will Blank(B), one will control(C), test tubes for SSF1 (T1 ,T2), SSF2 (T3,T4), test tubes for SmF1(T1*,T2*),SmF2(T3*,T4*). After adding 0.5ml buffered substrate + 0.5ml crude Enzyme and 1ml buffered substrate to control (C). . Then all the test tubes were incubated for 20min in RT. Then 1ml of NaOH

and 1ml of DNS were added into all test tubes. After that all the test tubes are boiled in water bath for 5-10 min. After cooling the test tubes, we added 5ml of water in every test tube and measured the O.D at 540nm in spectrophotometer.

Result and Discussion:-

OD SSF OD SmF

Sl No	O.D 540nm	Net O.D (T-C)	Average O.D		Sl No	OD 540nm	Net OD (T-C)	Average OD
B	0	------	------		B	0	------	------
C	0.073	------	------		C	0.024	------	------
T1	0.586	0.513	0.510		T1*	0.330	0.306	0.268
T2	0.581	0.508			T2*	0.255	0.231	
T3	0.962	0.889	0.910		T3*	0.426	0.426	0.496
T4	1.004	0.931			T4*	0.591	0.567	

From the standard curve of maltose, 0.1 OD = 0.14mg of maltose

= 342.3 X 0.14 /1000 (molecular weight of maltose 342.3g/mol) = 0.047 µmol

Calculation of amylase activity(V_0) from the standard curve of maltose – As we know 0.1 OD = 0.047µmol So, 1 OD =0.47µmol,V_0(SSF1) = 0.47 ×0.510/20 µmol/min= 0.011 µ mol/min
As the samecalculation,V_0'(SSF2)=0.021µmol/min

Calculation of amylase activity of SmF crude (V_0) from the standard curve of maltose – As we know 0.1 OD = 0.047µmol So, 1 OD =0.47µmol,
V_0(SmF1) = 0.47 × 0.268/20 µmol/min = 0.006 µ mol/min
As the same calculation,V_0'(SmF2) = 0.011µmol/min

In Room temperature after 20 min incubation it was found that the Amylase produced by SSF is more active than Amylase produced by SmF.

Characterization of the produced crude enzyme(Amylase):-

We characterized the newly produced enzyme from SSF and SmF based on different Temperature, pH and Thermal stability .

a)Characterization based on Temperature :- To characterize we used different condition of temperature of – 300C, 400C , 500C, 600C, 700 C . To estimate Amylase activity, We incubated crude enzymes produced from SSF and SmF at specific temperature of 300C, 400C, 500C, 600C,700C for 20 min., Then we measured the O.D value at 540 nm to check the enzyme activity at different temperature.

Result and Discussion:-

Sl No	**ODat30^{0}C 540nm**	**Net OD**
B	0	
C	0	
T1 30^o	0.763	0.942
T2 30^0	1.122	
T3 30^0	0.544	0.554
T4 30^0	0.565	

Calculation of amylase activity (V_0) at 30^0 C temperature:- From the standard curve of maltose – As we know 1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 0.942/20 µmol/min = 0.022 µmol/min
As the same calculation, V_0'(SmF)=0.013 µmol/min

Sl No	OD at40^{0}C 540nm	Net OD
B	0	
C	0	
T1 40^o	1.206	1.209
T2 40^0	1.213	
T3 40^0	0.638	0.685
T4 40^0	0.732	

Calculation of amylase activity (V_0) at 40^{0}C temperature:-From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 1.209/20 µmol/min = 0.028 µmol/min
As the same calculation, V_0'(SmF)=0.016 µmol/min

Sl No	OD at50^{0}C 540nm	Net OD
B	0	
C	0	
T1 50^o	1.198	1.231
T2 50^0	1.265	
T3 50^0	0.754	0.762
T4 50^0	0.771	

Calculation of amylase activity (V_0) at 50^{0}C temperature:- From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 1.231/20 µmol/min = 0.028 µmol/min
As the same calculation, V_0'(SmF)=0.017 µmol/min

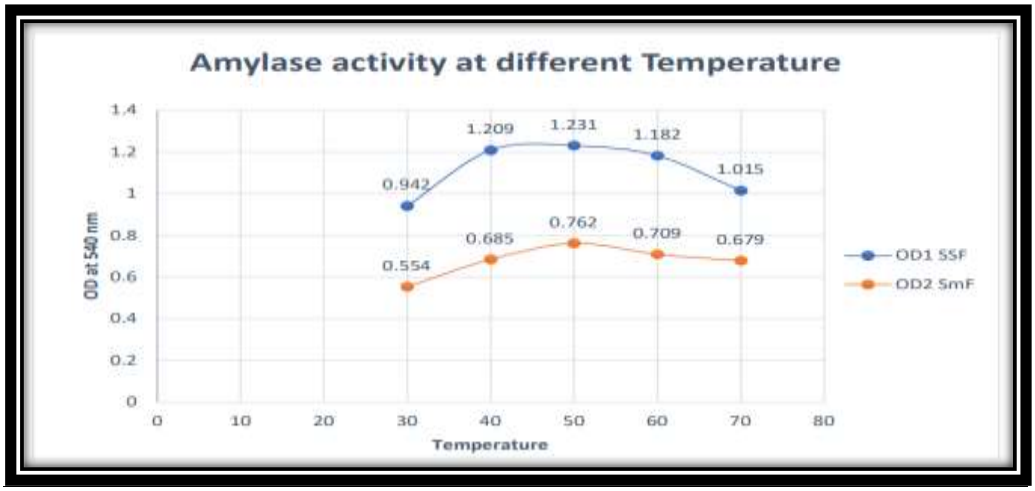
Sl No	OD at 60^{0}C 540nm	Net OD
B	0	
C	0	
T1 60^o	1.031	1.182
T2 60^0	1.333	
T3 60^0	0.706	0.709
T4 60^0	0.712	

Calculation of amylase activity (V_0) at 60^{0}C temperature:- From the standard curve of maltose –As we know
1 OD =0.47µmol,
V0'(SSF) = 0.47 × 1.182/20 µmol/min = 0.027 µmol/min
As the same calculation, V_0'(SmF)=0.016 µmol/min.

Sl No	OD at 70^{0}C 540nm	Net OD
B	0	
C	0	
T1 70^o	1.069	1.015
T2 70^0	0.962	
T3 70^0	0.687	0.679
T4 70^0	0.671	

Calculation of amylase activity (V_0) at 70^{0}C temperature:-
From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 1.015/20 µmol/min = 0.023 µmol/min
As the same calculation, V_0'(SmF)=0.015 µmol/min.

Graphical presentation of amylase activity based on the different temperature:-



As we can see from the OD value, the optimum temperature of amylase activity is 50^0 C. And the amylase from SSF is more active than the crude enzyme produced from SmF.

Note – [T1,T2] is SSF and [T3,T4] is SmF .

b) Characterization based on effect of different PH :- To characterize, activity of amylase in the different PH(4.5, 7.0, 9.5) , we used Acetic acid buffer (0.1 M ,PH 4.5) for PH 4.5 , Phosphate buffer (0.1 M PH7) for PH7 , Phosphate buffer(0.1M PH9.5) for PH 9.5.

To define Amylase activity, We incubated crude enzymes produced from SSF and SmF at specific PH of 4.5, 7.0, 9.5 for 20 min .

Then we measured the OD value at 540 nm ,at RT to determine the enzyme activity at different PH.

Result and Discussion:-

Sl No	OD at PH4.5 540nm	Net OD
B	0	
C	0	
T1 PH4.5	1.478	1.453
T2 PH4.5	1.429	
T3 PH4.5	0.651	0.659
T4 PH4.5	0.667	

Calculation of amylase activity (V_0) at PH 4.5:-
From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 1.453/20 µmol/min
= 0.034 µmol/min
As the same calculation, V_0'(SmF)=0.015 µmol/min.

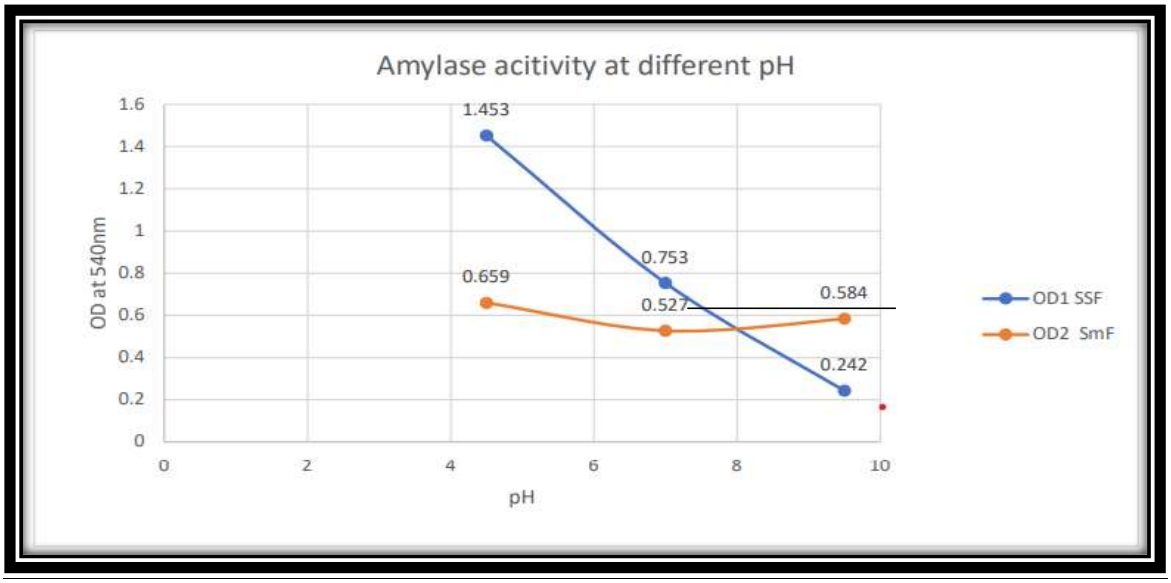
Sl No	OD at PH7.0 540nm	Net OD
B	0	
C	0	
T1 PH7.0	0.756	0.753
T2 PH7.0	0.751	
T3 PH7.0	0.545	0.527
T4 PH7.0	0.509	

Calculation of amylase activity (V_0) at PH 7.0:-
From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 0.753/20 µmol/min
= 0.017 µmol/min
As the same calculation, V_0'(SmF)=0.012 µmol/min.

Sl No	OD at PH7.0 540nm	Net OD
B	0	
C	0	
T1 PH7.0	0.756	0.753
T2 PH7.0	0.751	
T3 PH7.0	0.545	0.527
T4 PH7.0	0.509	

Calculation of amylase acivity (V_0) at PH 9.5:-
From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 0.242/20 µmol/min
= 0.005 µmol/min
As the same calculation,V_0'(SmF)=0.013 µmol/min.

Graphical presentation of amylase activity based on the effect of different PH :-



As we can see from the graph of OD value at different PH , the optimum PH for enzyme activity is PH 4.5. and the crude enzyme from SSF is more active than SmF.

Note – [T1,T2] is SSF and [T3,T4] is SmF .

c) Characterization based on Thermal stability :-To characterized based on thermal stability we measured enzyme activity at different Temperatureof 40^0 , 50^0, 60^0, 70^0 C .

To define Amylase activity, We incubated crude enzymes produced from SSF and SmF at specific temperature of 40^0, 50^0, 60^0,70^0 for 30 min.

Then we measured the OD value at 540 nm to check the enzyme activity at different temperature, to determine thermal stability.

Result and Discussion:-

Sl No	OD at40^0C 540nm	Net OD
B	0	
C	0	
T1 40^o	0.192	0.224
T2 40^0	0.257	
T3 40^0	0.748	0.742
T4 40^0	0.737	

Calculation of amylase activity (V_0) at 40^0 C temperature:- From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 0.224/30 µmol/min
= 0.003 µmol/min
As the same calculationV_0'(SmF)=0.011 µmol/min

Sl No	OD at50^{0}C 540nm	Net OD
B	0	
C	0	
T1 50º	0.460	0.541
T2 50^0	0.622	
T3 50^0	0.822	0.821
T4 50^0	0.820	

Calculation of amylase activity (V_0) at 50^0 C temperature:- From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 0.541 /30 µmol/min
= 0.008 µmol/min
As the same calculation, V_0'(SmF)=0.012 µmol/min

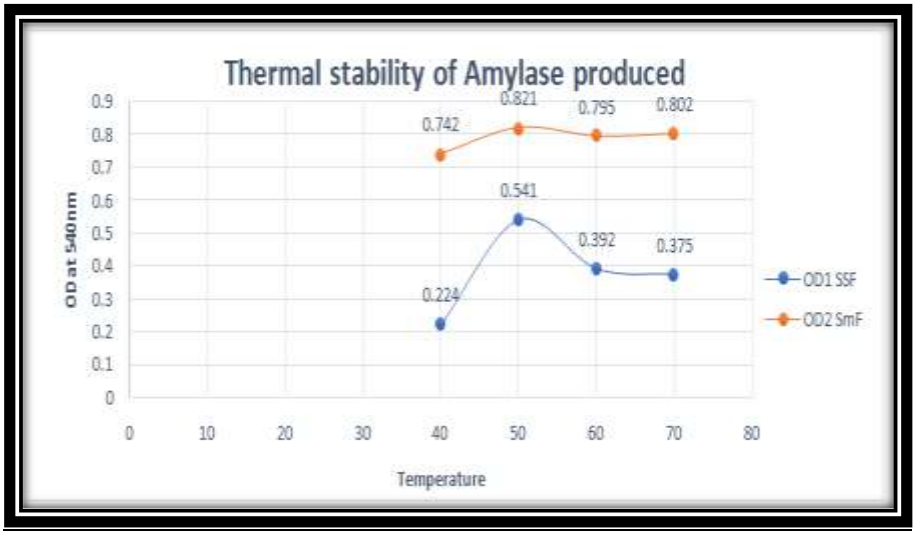
Sl No	OD at60^{0}C 540nm	Net OD
B	0	
C	0	
T1 60º	0.435	0.392
T2 60^0	0.350	
T3 60^0	0.800	0.795
T4 60^0	0.790	

Calculation of amylase activity (V_0) at 60^0 C temperature:- From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 0.392 /30 µmol/min
= 0.006 µmol/min
As the same calculation,V_0'(SmF)=0.012 µmol/min

Sl No	OD at70^{0}C 540nm	Net OD
B	0	
C	0	
T1 70º	0.357	0.375
T2 70^0	0.393	
T3 70^0	0.795	0.802
T4 70^0	0.810	

Calculation of amylase activity (V_0) at 70^0 C temperature:- From the standard curve of maltose – As we know
1 OD =0.47µmol,
V_0'(SSF) = 0.47 × 0.375 /30 µmol/min
= 0.005 µmol/min
As the same calculation, V_0'(SmF)=0.012 µmol/min

Graphical presentation of amylase activity based on the different temperature(Thermal stability):-



As we can see from the graph the Amylase has limited resistance to heat.. The enzyme displayed considerable stability up to 50°C. The Thermal stability of crude enzyme from SmF is more stable than SSF.

Note – [T1,T2] is SSF and [T3,T4] is SmF .

Conclusion:-

The production of amylase from *Aspergillus niger* was successfully carried out, and the enzyme was characterized based on different pH values, temperatures, and thermal stability. The experiment aimed to understand the behavior of the enzyme under various conditions to determine its optimal working parameters.

In terms of temperature, the enzymatic activity of amylase from *Aspergillus niger* showed an increase with rising temperatures until reaching an optimum at 50°C. This indicates that the enzyme is moderately thermostable and capable of functioning efficiently under moderate temperatures. However, above the optimal temperature, the enzyme activity declined rapidly, indicating a loss of stability and denaturation of the enzyme structure. At lower temperatures, the enzyme exhibited reduced activity, suggesting that it is less active under cold conditions. And also the activity of enzyme from SSF is more active than SmF.

Regarding pH, the results revealed that amylase from *Aspergillus niger* exhibited maximum activity at pH 4.5. At this pH, the enzyme demonstrated the highest enzymatic activity, indicating its preference for slightly acidic conditions. However, the enzyme activity decreased gradually as the pH deviated from the optimum, both towards acidic and alkaline pH values. This suggests that the enzyme is sensitive to changes in pH and its activity is significantly affected by extreme pH conditions.And also the activity of enzyme from SSF is more active than SmF.

Regarding thermal stability, the results indicated that amylase from *Aspergillus niger* has limited resistance to heat. The enzyme displayed considerable stability up to 50°C, as it retained a significant portion of its initial activity. However, prolonged exposure to higher temperatures resulted in a rapid decline in enzyme activity, indicating thermal inactivation. This suggests that the enzyme is not well-suited for applications requiring high-temperature processes.And also the activity of enzyme from SSF is more active than SmF.

In conclusion, amylase produced from *Aspergillus niger* showed optimal activity at pH 4.5 and a temperature of 50°C. However, the enzyme exhibited sensitivity to extreme pH values and limited thermal stability, being prone to denaturation and inactivation at higher temperatures. These findings are crucial for understanding the optimal conditions for utilizing amylase from *Aspergillus niger* in various industrial processes, such as food and starch processing, where specific pH and temperature ranges are required for efficient enzymatic activity. Further research could focus on enhancing the enzyme's stability and performance under extreme conditions to broaden its potential applications.

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